# EXHIBIT O

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# Comparative Tumorigenicity and DNA Methylation in F344 Rats by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*-Nitrosodimethylamine[1]

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## ABSTRACT

The tumorigenic activities and DNA methylating abilities in F344 rats of the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and the structurally related nitrosamine *N*-nitrosodimethylamine (NDMA) were compared. Groups of 30 male rats were given 60 s.c. injections of 0.0055 mmol/kg of either NNK or NDMA over a 20-week period (total dose, 0.33 mmol/kg). The experiment was terminated after 104 weeks. The numbers of rats with tumors were as follows for NNK and NDMA, respectively: liver, 10 and 6; lung 13 and 0; and nasal cavity, 6 and 1. NNK was significantly more tumorigenic than was NDMA toward the lung ($P < 0.01$) and nasal cavity ($P < 0.05$). Groups of rats were treated with a single s.c. injection of 0.39 mmol/kg or 0.055 mmol/kg of NNK or NDMA and the levels of 7-methylguanine and $O^6$-methylguanine were measured in liver, lung, and nasal mucosa 1–48 h after treatment. In liver and lung, levels of 7-methylguanine and $O^6$-methylguanine in DNA were 3–22 times ($P < 0.001$) greater in NDMA treated rats than in NNK treated rats. Levels of methylation induced by NDMA and NNK in the nasal mucosa were similar. The results of this study demonstrate that NNK is a more potent tumorigen than NDMA in the F344 rat and suggest that DNA methylation alone does not account for its strong tumorigenicity in rat lung and nasal mucosa.

## INTRODUCTION

Since the initial studies demonstrating the formation of NNK[3] from nicotine, its presence in tobacco, and its carcinogenicity (1–3), extensive investigations have shown that the amounts of NNK in tobacco and tobacco smoke are relatively high and that it is a strong carcinogen in rats, mice, and hamsters (4). NNK is believed to be one of the most important compounds responsible for cancer induction associated with tobacco use.

NNK is an *N*-methyl-*N*-nitrosamine, structurally related to NDMA (Fig. 1). Metabolism studies of NNK in the rat demonstrated that hydroxylation of the methylene carbon adjacent to the *N*-nitroso group occurred and, based on this, it was predicted that NNK, like NDMA, should be metabolized to methyl diazohydroxide and should cause DNA methylation *in vivo* (5). Three recent studies have shown that NNK does methylate DNA *in vivo* (6–8). Since extensive investigations of NDMA tumorigenicity and DNA methylation have been reported (9), and both NDMA and NNK are methylating agents, we compared their tumorigenic activities and DNA methylating abilities in order to gain further insight into the mechanism by which NNK induces tumors.

## MATERIALS AND METHODS

### Chemicals

NNK was synthesized (10) and NDMA was obtained from Aldrich Chemical Co. (Milwaukee, WI). Their purities were >99%. Trioctanoin was obtained from Eastman Kodak Company (Rochester, NY) and was redistilled before use.

### Bioassay

Male F344 rats, age 8 weeks, were purchased from Charles River Breeding Laboratories (Kingston, NY). They were allowed free access to NIH-07 diet and tap water. The rats were housed in groups of 3 in solid bottom polycarbonate cages with hardwood bedding and were kept under standard conditions [20 ± 2° (SD); 50 ± 10% relative humidity; 12-h light, 12-h dark cycle]. At 9 weeks of age, s.c. injections of either trioctanoin or of NNK (1.15 mg/kg, 0.0055 mmol/kg) or NDMA (0.41 mg/kg, 0.0055 mmol/kg) in trioctanoin began. Each group consisted of 30 rats which were given injections 3 times weekly for 20 weeks. The total doses were 0.33 mmol/kg of each nitrosamine. After the injections were complete, rats were observed until moribund. The experiment was terminated after 104 weeks. At autopsy, representative samples of all major organs were processed for histopathological examination (3, 11). Tumor incidence in the NNK and NDMA groups was compared using the $\chi^2$ test.

### DNA Methylation Study

**Animal Treatments.** Groups of 3 male F344 rats (220–250 g) were given a single s.c. injection of either NNK (81 mg/kg, 0.39 mmol/kg) or NDMA (29 mg/kg, 0.39 mmol/kg) in trioctanoin and were sacrificed at various intervals as summarized in Table 1. DNA was isolated from the liver and lungs of each rat and from the pooled nasal mucosa of 3 rats.

Groups of 6 male F344 rats were given a single s.c. injection of either NNK (11 mg/kg, 0.055 mmol/kg) or NDMA (4.1 mg/kg, 0.055 mmol/kg) in trioctanoin and sacrificed 4 or 24 h later. DNA was isolated from the liver of each rat and from the pooled nasal mucosa of 6 rats.

For the comparative studies of s.c. and oral administration, groups of 5 rats each were treated by gavage with either NNK or NDMA (0.39 mmol/kg) in 0.9% saline and sacrificed 4 h later. DNA was isolated from the liver and lungs of each rat and from the pooled nasal mucosa of 5 rats.

**DNA Isolation.** DNA isolation was carried out by the modified Marmur method (12), with slight modifications for the nasal mucosa DNA. Nasal septa with the attached mucosa were combined and homogenized in 3 ml sodium citrate buffer, pH 7.0. The homogenate was centrifuged at

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NDMA, *N*-nitrosodimethylamine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-butan-1-ol; $m^7$Gua, 7-methylguanine; $O^6$mGua, $O^6$-methylguanine.

10,000 × *g* for 30 min, and the supernatant was discarded. The precipitate was dispersed in 1.5 ml 1 M NaCl to which 10 μl of 15% sodium dodecyl sulfate solution was added. After cooling for 30 min at 0°C, the solution was extracted for 15 min with 1 ml $CHCl_3$/isoamyl alcohol (5/1, v/v). The resulting mixture was subjected to centrifugation at 10,000 × *g* for 15 min. The aqueous layer was removed and extracted again with $CHCl_3$/isoamyl alcohol. After centrifugation and separation of the layers, the aqueous phase was incubated for 30 min at room temperature with RNAase type III-A from bovine pancreas, 20 μl, and saturated aqueous sodium acetate, 5 μl. The solution was extracted with $CHCl_3$/isoamyl alcohol and, after separation of the layers, the DNA was precipitated by addition of 3 ml of ice cold ethanol. The DNA was washed several times in cold ethanol and acetone and then dried under $N_2$.

**Analysis of $m^7$Gua, $O^6$mGua, and Guanine in DNA.** The method was similar to that described previously (13). For analysis of liver DNA samples, 3–4 mg DNA was dissolved in 10 mM sodium cacodylate buffer, pH 7.0 (200 μl/mg DNA). The samples were hydrolyzed at 100°C for 35 min and then cooled to 0°C. After centrifugation the hydrolysate was divided into 2 portions of approximately equal volume. The apurinic DNA was precipitated from each portion by addition of enough ice cold 1 N HCl to bring the final concentration to 0.1 N HCl. In the portion to be used for analysis of $m^7$Gua, the precipitate was pelleted by centrifugation, and the volume of the supernatant was recorded. The second portion was used for analysis of $O^6$mGua and guanine. It was hydrolyzed at 80°C for 30 min and then cooled to 0°C. After centrifugation, the volume was measured. All samples were analyzed within 12 h of hydrolysis.

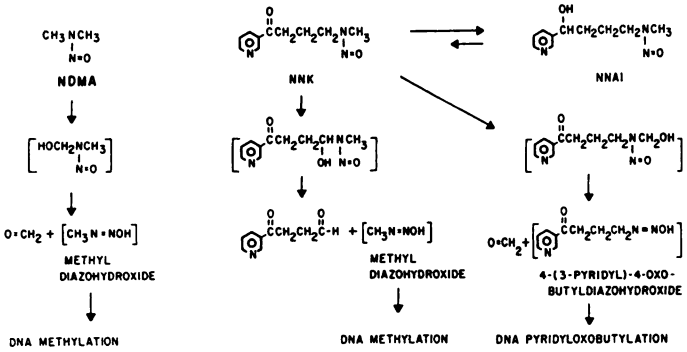


Fig. 1. Pathways of metabolism of NDMA or NNK leading to DNA methylation or DNA pyridyloxobutylation.

For analysis of lung and nasal mucosa DNA, 0.3–1.5 mg DNA was dissolved in 0.2–0.3 ml of 10 mM sodium cacodylate buffer. Hydrolyses were carried out as described above, except that the sample was not divided into 2 portions.

Quantitative analysis was performed by high-performance liquid chromatography with UV and fluorescence detection. The system consisted of a WISP 710B automatic injector and Model 510 solvent delivery system (Millipore, Waters Chromatography Division, Milford, MA) coupled to a Partisil 10 SCX column (Whatman, Inc., Clifton, NJ), a Model 440 absorbance detector (Millipore), and a Model 650-10S fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Aliquots of 0.1 ml containing 0.05–0.15 μmol guanine (liver and lung) or 0.02–0.10 μmol guanine (nasal mucosa) were analyzed. The column was eluted with 0.03–0.05 M ammonium phosphate buffer, pH 2.0, at 2 ml/min. Guanine was measured by UV absorbance at 254 nm. Levels of $m^7$Gua and $O^6$mGua were measured by fluorescence detection with settings as follows: excitation, 280 nm; and emission, 366 nm. The detection limits were approximately 10 and 0.5 pmol/injection of $m^7$Gua and $O^6$mGua, respectively. Quantification was carried out using standard curves constructed for each analysis. Statistical significance was determined using the Mann-Whitney rank test.

**Levels of NNK and NDMA in Blood.** Two groups of 5 male F344 rats were given a single s.c. injection of either NNK or NDMA (0.39 mmol/kg) in trioctanoin. Four h later, blood was collected by cardiac puncture and analyzed for NNK and its major metabolite NNAL by combined gas chromatography-thermal energy analysis as described previously (14). The injection sites were also extracted and analyzed.

## RESULTS

The comparative bioassay of NNK and NDMA was terminated after 104 weeks, when overall mortality had reached 65%. Weight curves and survival curves were not significantly different among the three groups. Tumor incidence is summarized in Table 2. The incidence of liver tumors was similar in the NNK and NDMA treated groups. However, NNK induced significantly

Table 1

*Levels of $m^7$Gua and $O^6$mGua in F344 rat tissues at intervals after s.c. injection of NNK or NDMA (0.39 mmol/kg)*

Groups of 3 male F344 rats were given an s.c. injection of NNK or NDMA in trioctanoin (0.39 mg/kg) and sacrificed at the intervals noted. Values are mean of duplicate determinations on DNA from liver and lung of each of 3 rats and mean of duplicate determinations on DNA pooled from the nasal mucosa of 3 rats.

| | | μmol/mol guanine | | | | | |
|---|---|---|---|---|---|---|---|
| | | Liver | | Lung | | Nasal mucosa | |
| Treatment | Survival interval (h) | $m^7$Gua | $O^6$mGua | $m^7$Gua | $O^6$mGua | $m^7$Gua | $O^6$mGua |
| NNK | 1 | 367 ± 27[a] | 26 ± 3.5 | ND[b] | ND | 1520 | 170 |
| NDMA | 1 | 1850 ± 230 | 230 ± 35 | 203 ± 29 | 15 ± 3 | 1580 | 168 |
| NNK | 4 | 817 ± 31 | 74 ± 5.2 | ND | 3 ± 3 | 1960 | 230 |
| NDMA | 4 | 7110 ± 300 | 980 ± 12 | 580 ± 62 | 71 ± 7 | 3470 | 380 |
| NNK | 12 | 935 ± 22 | 108 ± 5 | ND[c] | 6.7 ± 0.1 | 2060 | 251 |
| NDMA | 12 | 7650 ± 340 | 1230 ± 47 | 635 ± 7 | 70 ± 6 | 3300 | 441 |
| NNK | 24 | 1107 ± 96 | 87 ± 7 | 62 ± 4.3 | 7.9 ± 1.2 | 1400 | 210 |
| NDMA | 24 | 6270 ± 150 | 1240 ± 20 | 523 ± 76 | 65 ± 15 | 2910 | 490 |
| NNK | 36 | 853 ± 96 | 51 ± 21 | 87 ± 24 | 8.9 ± 0.2 | 1220 | 190 |
| NDMA | 36 | 3450 ± 500 | 750 ± 90 | 400 ± 26 | 60 ± 11 | 2050 | 400 |
| NNK | 48 | 559 ± 14 | 18 ± 3 | ND | 5.7 ± 0.7 | 589 | 142 |
| NDMA | 48 | 2020 ± 143 | 400 ± 21 | 318 ± 20 | 62 ± 4 | 1620 | 311 |

[a] Mean ± SD.
[b] ND, not detected.
[c] $m^7$Gua was obscured by another peak.

Table 2

*Induction of tumors by NNK and NDMA in F344 rats*

Rats were given s.c. injections of NDMA or NNK in trioctanoin 3 times weekly for 20 weeks; total doses of each nitrosamine b.w. were 0.33 mmol/kg. The experiment was terminated after 104 weeks.

| | | No. of rats with tumors[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Liver | | Lung[c] | | Nasal cavity[d] | |
| Group | Effective no. of rats[a] | Hepatocellular carcinoma | Adenoma | Adenocarcinoma | Adenoma | Squamous cell carcinoma | Squamous cell papilloma |
| NNK | 27 | 2 | 8 | 4 | 9 | 1 | 5 |
| NDMA | 27 | 2 | 4 | 0 | 0 | 0 | 1 |
| Vehicle | 26 | 0 | 0 | 0 | 1 | 0 | 0 |

[a] Number of rats autopsied.
[b] Other tumors: Leydig tumors, NDMA group, 21; NNK group, 22; vehicle control group, 20; abdominal mesothelioma, NDMA group, 2; NNK group, 2; vehicle control, 0; s.c. sarcoma, NDMA group, 5; NNK group, 2; vehicle control group, 2; prostate *in situ* carcinoma, NDMA group, 4; NNK group, 3; vehicle control group, 1; leukemia/lymphoma, NDMA group, 4; NNK group, 3; vehicle control group, 4.
[c] Tumor incidence in NNK group > NDMA group, $P < 0.01$.
[d] Tumor incidence in NNK group > NDMA group, $P < 0.05$.

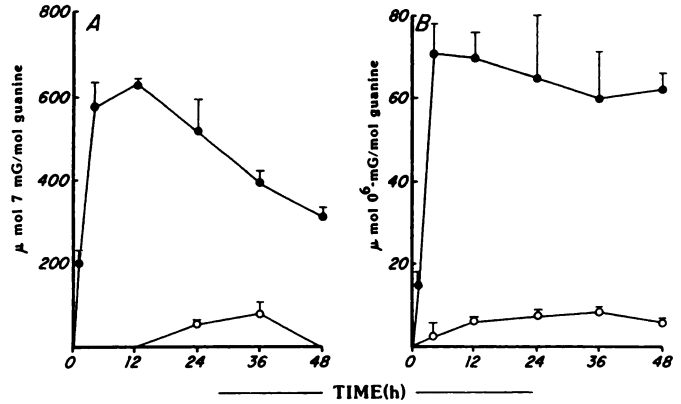


Fig. 2. Comparative levels of (*A*) $m^7Gua$ and (*B*) $O^6mGua$ in lung DNA at intervals after treatment with NDMA (0.39 mmol/kg) (●) or NNK (0.39 mmol/kg) (○).

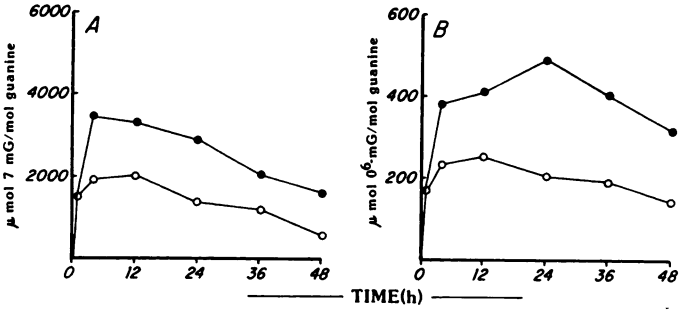


Fig. 3. Comparative levels of (*A*) $m^7Gua$ and (*B*) $O^6mGua$ in nasal mucosa DNA at intervals after treatment with NDMA (0.39 mmol/kg) (●) or NNK (0.39 mmol/kg) (○).

Table 3

*Levels of $m^7Gua$ and $O^6mGua$ in F344 rat tissues after p.o. treatment with NNK or NDMA (0.39 mmol/kg)*

Groups of 5 male F344 rats were gavaged with NNK or NDMA (0.39 mmol/kg) in 0.9% aqueous NaCl and sacrificed 4 h later. Values are mean for DNA from each of 5 rats (liver and lung) or mean of pooled DNA from nasal mucosae of 5 rats.

| | μmol/mol guanine | | | | | |
|---|---|---|---|---|---|---|
| | Liver | | Lung | | Nasal mucosa | |
| Group | $m^7Gua$ | $O^6mGua$ | $m^7Gua$ | $O^6mGua$ | $m^7Gua$ | $O^6mGua$ |
| NNK | 1400 ± 222[a] | 155 ± 6 | 53 ± 10 | 3.5 ± 0.9 | 2090 | 235 |
| NDMA | 11300 ± 1220 | 1390 ± 149 | 1060 ± 214 | 90 ± 8 | 3270 | 333 |

[a] Mean ± SD.

Table 4

*Levels of $m^7Gua$ and $O^6mGua$ in F344 rat tissues at intervals after s.c. injection of NNK or NDMA (0.055 mmol/kg)*

Groups of 6 male F344 rats were given a s.c. injection in trioctanoin and sacrificed after 4 or 24 h. Values are mean either for 3 rats (liver) or from pools of 6 nasal mucosa DNA samples.

| | | μmol/mol guanine | | | |
|---|---|---|---|---|---|
| | | Liver | | Nasal mucosa | |
| Treatment | Survival interval (h) | $m^7Gua$ | $O^6mGua$ | $m^7Gua$ | $O^6mGua$ |
| NNK | 4 | 190 ± 14[a] | 16 ± 2 | 340 | 33 |
| NDMA | 4 | 1420 ± 79 | 170 ± 21 | 280 | 9 |
| NNK | 24 | 99 ± 28 | 4 ± 1 | ND[b] | 20 |
| NDMA | 24 | 680 ± 12 | 45 ± 17 | ND | 19 |

[a] Mean ± SD.
[b] ND, not detected.

more lung tumors ($P < 0.01$) and nasal cavity tumors ($P < 0.05$) than did NDMA. The histopathology of the tumors induced by NNK was similar to that described previously (3, 11).

Four h after s.c. injection of NNK, levels of NNK and NNAL in blood were 2.3 ± 1.0 and 18.7 ± 10.2 nmol/ml, respectively. In contrast, NDMA was not detected (<0.037 nmol/ml). Less than 0.5% of the doses of either NNK or NDMA remained at the injection site.

Levels of $m^7Gua$ and $O^6mGua$ in DNA of liver, lung, and nasal mucosa 1–48 h after treatment with a single s.c. injection of NNK or NDMA (0.39 mmol/kg) are summarized in Table 1 and, for lung and nasal mucosa, in Figs. 2 and 3. In liver and lung, NDMA methylation was significantly greater ($P < 0.001$) than was NNK methylation at all intervals. In nasal mucosa, NDMA methylation exceeded NNK methylation, and the differences were not as great as were those observed in liver and lung.

NNK methylation was most extensive in the nasal mucosa, followed by the liver and lung. NDMA methylation was greatest in the liver, followed by the nasal mucosa and lung. Similar results were obtained when the compounds were administered by gavage in saline (Table 3).

Table 4 summarizes the levels of DNA methylation by NDMA and NNK in liver and nasal mucosa, 4 and 24 h after s.c. injection of 0.055 mmol/kg of each nitrosamine, which is one-seventh the dose used in Table 1. As in the higher dose study, NDMA methylation greatly exceeded NNK methylation in the liver. Levels of methylation in the nasal mucosa were similar. Levels of methylation in the lung were below the detection limit.

## DISCUSSION

The incidence of nasal cavity and lung tumors in the rats treated with NNK agrees well with expectations based on previous bioassays of NNK (3, 15). A total dose of NNK (1.0 mmol/kg), given by the same protocol as that used in the present study, induced nasal cavity tumors in 20 of 27 male F344 rats and lung tumors in 23 of 27 rats (15). The present results extend these findings and again demonstrate the remarkable ability of NNK to induce lung tumors in rodents (4).

In 1956, Magee and Barnes (16) first reported that NDMA was a hepatocarcinogen in the rat. Since then, over 300 nitrosamines have been shown to cause tumors in 40 animal species, but the role of nitrosamines in human cancer has remained an open question (9, 17). Recently however a growing consensus has developed supporting the idea that NNK and the related tobacco specific nitrosamines are causative agents in human cancer (4, 18, 19). This consensus is based on epidemiological studies which show an association between snuff-dipping and the incidence of oral cancer and on extensive analytical data which have shown that NNK and the other tobacco specific nitrosamines are the major known carcinogens present in snuff (4, 20). In addition, the ability of NNK to induce lung tumors suggests that, among the many carcinogens in tobacco smoke, it may play an important role in lung cancer induction. The present results further support the view that NNK is a potential human carcinogen. Extensive large scale bioassays of NDMA administered to rats in the drinking water have shown that its hepatocarcinogenicity was clearly observable at a daily dose of 0.02 mg/kg body weight, corresponding to a total dose of approximately 20 mg/kg (0.3 mmol/kg), which is the same as the total dose used in the present study (21). Although the lower limits of NNK tumorigenicity in rats have yet to be established, its tumorigenicity compared to that of NDMA suggests that its effects should be measurable at doses considerably lower than 0.3 mmol/kg. The lifetime exposure of a snuff-dipper to NNK can be estimated as approximately 0.02 mmol/kg, based on daily use, for 30 years, of 10 g of snuff containing 3.3 ppm of NNK. The present results, which show that NNK is more tumorigenic than is NDMA in the F344 rat, together with the available data on NDMA tumorigenicity, indicate that such exposures may be sufficient to induce cancer.

The results of the comparative DNA methylation study clearly show that, independent of the route of administration or dose, levels of $m^7$Gua and $O^6$mGua formed in liver and lung upon treatment with NDMA greatly exceeded those formed from NNK. DNA methylation by NDMA also exceeded DNA methylation by NNK in the nasal mucosa at the higher dose, but at the lower dose the levels of DNA methylation by NDMA and NNK were similar. There are some limitations to this comparative DNA methylation study. First, the doses used were 70-fold (Table 1) and 10-fold (Table 4) greater than the single doses in the tumorigenicity study. Second, only a single dose was used, whereas multiple doses were used in the tumorigenicity experiment. Third, only $m^7$Gua and $O^6$mGua were measured. Although $O^6$mGua is known to have miscoding properties, other lesions such as $O^4$-methylthymidine may be involved in the initiation of tumorigenesis (22). Fourth, DNA methylation was measured in whole tissues rather than in individual cell types. Nevertheless, levels of $O^6$mGua formation by NNK and NDMA do not correlate with their relative tumorigenic activities, at least during the 48-h period studied. NNK was more tumorigenic than was NDMA in the lung, but the levels of $O^6$mGua in lung upon treatment with NDMA were 7–22 times greater than those caused by NNK. NNK was more tumorigenic than was NDMA in the nasal mucosa, but the levels of $O^6$mGua caused by the two nitrosamines were similar. In liver, NNK and NDMA had similar tumorigenic activities, but levels of $O^6$mGua upon treatment with NDMA were 9–22 times greater than upon treatment with NNK. These results suggest that factors other than or in addition to $O^6$mGua formation may be involved in NNK tumorigenesis. Metabolic studies of NNK have shown that hydroxylation of the methyl group occurs *in vitro* and *in vivo* (5, 23). This produces formaldehyde and, most likely, 4-(3-pyridyl)-4-oxobutyldiazohydroxide (Fig. 1). Studies with a stable precursor to the latter have shown that it is highly mutagenic (10), and a product of its interaction with deoxyguanosine, 2′-deoxy-*N*-[1-methyl-3-oxo-3-(3-pyridyl)propyl]guanosine, has recently been identified (24). Experiments aimed at the characterization of this adduct in DNA of rats treated with NNK are in progress. We suggest that a combination of DNA methylation and DNA pyridyloxobutylation is critical in the initiation of tumors by NNK.

Recently, Belinsky *et al.* (25) have measured the levels of DNA methylation by NNK in rat liver, lung, and nasal mucosa during 12 days of NNK treatment. They observed an accumulation of $O^6$mGua in the lung. Their results appear to be consistent with ours because, as shown in Fig. 2, $O^6$mGua formed in lung from NDMA or NNK may persist. Based on their results, Belinsky *et al.* suggested that the accumulation of $O^6$mGua in lung DNA was important in the induction of respiratory tumors by NNK. However, Fig. 2 suggests that $O^6$mGua may also accumulate in lung DNA upon chronic treatment with NDMA, but no lung tumors were observed. As suggested above, factors in addition to $O^6$mGua formation may be involved in the induction of lung tumors by NNK.

The levels of $m^7$Gua and $O^6$mGua in the hepatic DNA of NDMA treated rats reached a maximum between 4–12 h and then declined at similar rates, probably as a consequence of repair. The observed levels of $m^7$Gua and $O^6$mGua over the 48-h period studied are in agreement with expectations based on previous reports (26, 27). In the rats treated with the higher dose of NNK, the decline between 24–48 h of $m^7$Gua in hepatic DNA was not as rapid as in the rats treated with the higher dose of NDMA. This suggests that methylation by NNK was still occurring between 24 and 48 h. Levels of $O^6$mGua in the hepatic DNA of NNK treated rats declined more rapidly between 12 and 48 h than in NDMA treated rats, probably due to more efficient repair of the lower initial amounts (27). In lung, formation of $m^7$Gua and $O^6$mGua was clearly slower in the NNK treated rats than in the rats treated with NDMA. Taken together, these results are consistent with previous studies on the metabolism of NDMA and NNK. Based on rates of exhalation of $^{14}CO_2$ in rats treated with doses of [$^{14}$C]NDMA similar to those used in the present study, it has been estimated that metabolism of NDMA and methylation of hepatic DNA is complete within 4–6 h (28). In contrast, NNK is converted to NNAL *in vivo* and NNAL persists in blood for at least 10 h (5, 19, 23). In this study, NNK and NNAL but not NDMA were detected in blood 4 h after treatment. The more rapid and extensive methylation of hepatic and lung DNA by NDMA compared to NNK is probably a consequence of more rapid and extensive $\alpha$-hydroxylation of NDMA than of NNK

and NNAL. $\alpha$-Hydroxylation of either carbon of NDMA gives a methylating agent, and it has been estimated that this pathway accounts for 40–60% of NDMA metabolism in hepatocytes (29). In contrast, only methylene hydroxylation of NNK or NNAL is known to lead directly to a methylating agent. In liver, hydroxylation of the methyl group, reduction of the carbonyl group, and oxidation of the pyridine nitrogen of NNK have been observed in addition to the requisite methylene hydroxylation leading to a methylating agent (5, 23).

In contrast to the results obtained in liver and lung, formation of $m^7$Gua and $O^6$mGua in nasal mucosa DNA of rats treated with NNK or NDMA occurred at similar rates and to similar extents. The relatively high methylating activity of NNK in the rat nasal mucosa is consistent with our previous studies which demonstrated that this tissue has exceptional activity for $\alpha$-hydroxylation of NNK (23, 30). Levels of NNAL formation and of pyridine-*N*-oxidation in rat nasal mucosa were relatively low (30). We are not aware of any previous studies on NDMA methylation of nasal mucosa DNA *in vivo*. However, our observations are consistent with the findings that rat nasal mucosa contains relatively high levels of cytochrome P-450 enzymes and that these enzymes can catalyze the $\alpha$-hydroxylation of NDMA (31, 32).

In summary, this study has shown that NNK is a more potent tumorigen than NDMA upon s.c. administration to F344 rats and has suggested that factors other than $O^6$mGua formation may be involved in its tumorigenicity in rat lung and nasal mucosa.

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